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(19)(CA) **CANADIAN PATENT** (12)

(54) Methods and Media for Enhanced Somatic Embryogenesis

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Abstract

Improved Methods and Media for Enhanced Somatic Embryogenesis

Methods and media are provided to improve the quantity and quality of the embryos obtained by induction of somatic plant tissue. Media provide for the addition of an amount of selected amino acids sufficient to increase the number of somatic embryos generated. The invention further provides for such media including sources of ammonium ion and methods for the culturing of plant somatic tissue.

Technical Field

This invention relates generally to the culturing of embryonic plant cells and tissue and more specifically to an improved medium particularly adapted for sustaining embryos produced by induction from somatic tissue *in vitro* and a method of using the medium.

Background Art

It has been recognized that recent progress in genetic engineering offers plant breeders the ability to avoid the delay in crop improvement inherent in classical breeding techniques. However, there remain difficulties in the application of these techniques, for unicellular and multicellular organisms require different procedures to change the entire genetic message. In microorganisms, one attempts to effect change at the cellular level with confidence that this will be reproduced through succeeding generations of cells.

In multicellular organisms, such as plants, it is advantageous to perform genetic manipulations at the cellular level, then regenerate and raise a mature plant expressing the new characteristic. Foreign genetic material can be incorporated into the host cell by, e.g., plasmid insertion to provide for specific changes or protoplast fusion to provide wholesale genetic manipulation. Further genetic manipulation using the techniques of traditional plant breeding with mature plants can be used to incorporate the new trait into agriculturally useful varieties. Allard, R.W., *Principles of Plant Breeding*, John Wiley and Sons, New York (1960); Simmons, N.W., *Principles of Crop Improvement*, Langman Group, Ltd., London, (1972).

In vitro cultivation of plant cells and tissue requires that the cultures be maintained in a medium which

provides nutrition and sustains viability. Examples of commonly used tissue culture media have been reviewed in Huang, L. and T. Murashige, "Plant Tissue Culture Media: Major constituents, their preparation, and some applications" Tissue Culture Association Manual 3:539-548. Tissue Culture Association, Rockville, M.D., (1977) and de Fossard, R.A. Tissue Culture for Plant Propagators, University of New England Printery, Armidale, N.S.W., Australia (1976). This culture maintenance can be promoted in plant organ, tissue, or cell cultures.

In somatic embryogenesis culture, somatic plant cells are typically induced to undergo repeated cell divisions on a nutritive culture medium substrate, producing an amorphous cell mass known as callus. The callus can be maintained through subculture to allow mass proliferation. The callus may also be induced to undergo differentiation, which produces the organized tissues and organs of the mature plant. Somatic embryos may also form in culture from other pre-existing embryos. The parent embryos may range from the immature globular stage to mature, germinating embryos. Lupotto, E., "Propagation of an embryonic culture of Medicago sativa L.", Zeit. Pflanzenphysiol. 111:95-104 (1983). Thus, somatic embryos may arise from undifferentiated callus or from pre-existing embryos in plant tissue culture.

In this manner, genetic changes may be affected on a cellular or embryo level and then maintained through subsequent development to produce an entire crop with identical genetic characteristics. This allows the plant breeder to bypass the normal genetic barriers in plant reproduction, and obtain a more uniform and advantageous field crop.

Using current technology, it is possible to produce thousands of plants from one gram of cells using

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the process of somatic embryogenesis. Evans, D.A., W.R. Sharp, and C.E. Flick, "Growth and behavior of cell cultures: embryogenesis and organogenesis", in Plant Tissue Culture - 1981, T.A. Thorpe, ed., Academic Press, pp. 45-113 (1981). These embryos could be germinated and transferred into the greenhouse or field where mature plants can develop. A plant breeder would use these plants to recover useful genetic variation or to clonally propagate varieties for use in a plant breeding program.

Certain techniques are known for determining the quantity and quality of the embryonic tissue obtained through culture of somatic plant parts. The quantity of somatic embryos can be measured by determining the yield of structures associated with the stages of development of the embryo. Fujimura, T., and A. Komamine, "Synchronization of somatic embryogenesis in a carrot cell suspension culture" Plant Physiology, 64:162-164 (1979); Verma, D.C. and D.K. Dougall, "Influence of carbohydrates on quantitative aspects of growth and embryo formation in wild carrot suspension cultures" Plant Physiology 59:81-85 (1977). This measurement is generally done by counting structures using a dissecting microscope.

Somatic embryo quality can be assessed by various methods. Embryo development is typically determined visually by searching for globular, heart, torpedo and plantlet stages. Ammirato, P.V., "The effects of abscisic acid on the development of somatic embryos from cells of caraway (Carum carri L.) "Botanical Gazette 135:328-337 (1974). Embryo development or quality can also be determined from the yield of plantlets obtained from individual somatic embryos. Drew, R.L.W., "The development of carrot (Daucus carota L.) embryoids (derived from cell suspension culture) into plantlets on a sugar-free basal medium" Horticultural Research 19:79

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(1979). However, plantlet formation is rarely measured despite its importance in determining the yield of functionally useful embryos for field use.

Techniques have been described for improving the quantity or yield of embryos. For example, formation of somatic embryos is known to require a source of ammonium in the culture medium for embryogenesis to occur. Halperin, W. and D.F. Wetherell, "Ammonium requirement for embryogenesis in vitro", Nature 205:519-520 (1965). Walker, K.A., and S.J. Sato, "Morphogenesis in callus tissue of Medicago sativa: the role of ammonium ion in somatic embryogenesis", Plant Cell Tissue Organ Culture 1:109-121 (1981). Since most plant cell culture media contain some ammonium, it has been considered important to adjust the ammonium concentration to an appropriate level to achieve high embryo yield and quality. See Walker and Sato, supra, and Wetherell, D.F. and D.K. Dougall, "Sources of nitrogen supporting growth and embryogenesis in cultured wild carrot tissue", Physiologia Plantarum 37:97-103 (1976).

In plant cell culture and in mature plants, ammonium and glutamine or glutamate can be readily interconverted by the cells. Ojima, K. and K. Ohira, "Nutritional requirements of callus and cell suspension cultures" in Frontiers of Plant Tissue Culture-1978, T.A. Thorpe, ed., University of Calgary Offset Printing Services, pg. 265-275 (1978); Miflin, B.J. and P.L. Lea, "Ammonium Assimilation" in The Biochemistry of Plants, P. Stumpf and E. Conn eds., Academic Press., Vol. 6, pg. 169-201 1980; Dougall, D.K. "Current problems in the regulation of nitrogen metabolism in plant cell cultures", in Plant Tissue Culture and its Biotechnological Application. W. Barz, E. Reinhard, and M.H. Zenk, eds., Springer-Verlag, Berlin, pg. 76-81 (1977).

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The perceived proximity of ammonium to glutamine and glutamate in plant metabolism is reflected by the protocols for systematic studies of the effects of amino acids on somatic embryogenesis. Studies using carrot cells have removed ammonium from the plant cell culture medium and tested single amino acids for their effect on somatic embryogenesis. Wetherell and Dougall, supra; Kamada, H. and H. Harada, "Studies on the organogenesis in carrot tissue cultures II. Effects of amino acids and inorganic nitrogenous compounds on somatic embryogenesis," Zeit. Pflanzenphysiologie 91:453-463 (1979). Wetherell and Dougall found that amino acid additions did not improve embryogenesis compared to ammonium ion. Kamada and Harada, on the other hand, found that alanine and glutamine act as good reduced nitrogen sources for embryogenesis in the absence of ammonium or in the absence of ammonium and nitrate. One report has recommended against the use of amino acid additions which were 20 mM or higher (Reinert, J. and M. Tazawa, "Wirkung von Stickstoffverbindungen und von Auxin auf die Embryogenese in Gewebekulturen," Planta 87:239 (1969), (Text in German).

The above studies indicate that there is an equivalence among sources of reduced nitrogen, such as ammonium and amino acids. Wetherell and Dougall, supra; Kamada and Harada, supra. The metabolic equivalence of ammonium and amino acids is further shown by the studies of Tazawa and Reinert (Tazawa, M. and Reinert, J., "Extracellular and intracellular chemical environments in relation to embryogenesis in vitro", Protoplasma 68:157-173 (1969)). In an investigation of the internal levels of ammonium in carrot cultures undergoing somatic embryogenesis, it was found that the level of ammonium correlated with the amount of embryogenesis in the culture

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regardless of whether cultures were fed ammonium, amino acid or nitrate. It was concluded that the internal level of NH_4^+ is the critical factor in stimulating somatic embryogenesis. The internal NH_4^+ level is derived from either externally supplied NH_4^+ or amino acids, or by the biological reduction of nitrate to NH_4^+. Internally, NH_4^+ can be converted to organic nitrogen compounds to supply amino acids for normal cell requirements, Tazawa and Reinert, supra. Hence amino acids are believed to act by releasing ammonium, which stimulates embryogenesis.

Factors which have been noted to improve embryo development are abscisic acid, zeatin, gibberellic acid, high sucrose concentrations and light. Ammirato, P.V. and F.C. Steward, "Some effects of environment on the development of embryos from cultured free cells", Botanical Gazette 132:149-158 (1971); Ammirato, P.V., "Hormonal control of somatic embryo development from cultured cells of caraway. Interactions of abscisic acid, zeatin and gibberellic acid", Plant Physiology 59:579-586 (1977). The effect of ammonium and amino acids on embryo quality is not known to have been recognized. Ammirato, P.V., "The regulation of somatic embryo development in plant cell cultures: Suspension culture techniques and hormone requirements", Bio/Technology 1:68-74 (1983).

In addition, conversion frequency, as a measure of embryo quality, has heretofore not been recognized or used systematically to improve embryo development. Embryo maturation has been determined by visual assessment of embryo morphology, but this method does not measure the frequency of plant formation from individual embryos.

Therefore it is an object of this invention to provide methods and materials to increase the quantity and quality of somatic embryos produced from plant tissue.

It is a further object of this invention to

provide optimized sources of reduced nitrogen for somatic embryogenesis.

It is yet another object of this invention to provide methods and materials allowing mass propagation of numerous species of plants through somatic embryogenesis.

It is a still further object of this invention to provide methods and materials for a generation of numerous viable somatic embryos with identical genetic and phenotypic traits.

Other objects, advantages, and features of the present invention will become apparent from the following description and the accompanying examples.

Disclosure of the Invention

This invention provides novel and improved methods and materials for producing numerous high quality somatic embryos from plant tissue by the addition of optimal amounts of amino acids and sources of reduced nitrogen. One aspect of the present invention provides a method for producing embryonic tissue from Gramineae, Umbelliferae or Malvaceae somatic tissue, wherein somatic tissue is regenerated from induced cells in a nutritive plant cell culture medium to form embryonic tissue, said method comprising:

providing a nutritive plant cell culture medium comprising:

a medium having all mineral salts, vitamins and nutrients required to maintain tissue viability, together with a source of ammonium ion; and

an addition to said medium of at least one amino acid component selected from the group consisting of L-proline, L-arginine, L-lysine, L-asparagine, L-serine, L-ornithine and the amide, alkyl ester and dipeptidyl derivatives thereof, upon which somatic embryos can

differentiate from induced cells, the ammonium and amino acid components of said medium provided in amounts sufficient to increase the quantity or improve the quality of somatic tissue to be produced;

exposing undifferentiated plant somatic tissue selected from the group consisting of Gramineae, Umbelliferae and Malvaceae somatic tissue to said culture medium to cause embryogenesis in said plant somatic tissue to form somatic embryos; and

sustaining said somatic embryos on said culture medium to permit embryo development into plantlets.

Another aspect of the present invention provides a method of producing embryonic tissue from Gramineae, Umbelliferae or Malvaceae somatic tissue, wherein somatic tissue is regenerated from induced cells in a nutritive plant cell culture medium to form embryonic tissue, said method comprising:

providing a nutritive plant cell culture medium comprising all mineral salts, vitamins and nutrients required to maintain tissue viability, which medium is substantially free of ammonium ion, together with an addition to said medium of L-glutamine and at least one amino acid component selected from the group consisting of L-proline, L-arginine, L-lysine, L-asparagine, L-serine, L-ornithine, the amide, alkyl ester and dipeptidyl derivatives thereof, upon which somatic embryos differentiate from induced cells, the amino acid components of said medium provided in amounts sufficient to increase the quantity or improve the quality of somatic tissue to be produced;

exposing undifferentiated plant somatic tissue selected from the group consisting of Gramineae, Umbelliferae and Malvaceae somatic tissue to said culture medium to cause embryogenesis in said plant somatic tissue

to form somatic embryos; and

sustaining said somatic embryos on said provided nutritive plant cell culture medium to thereby permit embryo development into plantlets.

Brief Description of the Drawing

The single drawing is a graphic representation of the increase in number of somatic embryos produced as a function of the concentration of amino acids added to the medium.

Best Mode for Carrying Out the Invention

The present invention provides methods for enhanced quantity and quality of embryos produced from plant somatic tissue by providing a medium for culturing said cells and tissue which contains a sufficient amount of selected amino acids to stimulate somatic embryogenesis.

The present invention also provides for such enhanced quantity and quality by providing a medium for culturing such cells and tissue which contains selected amino acids together with sources of ammonium ion in amounts sufficient to stimulate the quantity and quality of somatic embryos. Also provided is a method for using such plant tissue culture medium.

While it has previously been believed that amino acids served as simple equivalents to the desired ammonium media component, it has surprisingly been found that amino acids can serve as replacement for ammonium ion which enhance the production of somatic embryos over the equivalent concentrations of ammonium. It has also been surprisingly found that selected amino acids together with an additional source of ammonium ion can provide substantially increased benefits which would not be

predicted from a simple additive effect of increased ammonium ion concentration.

It has been found that a medium which contained an amino acid selected from the group consisting of proline, arginine, lysine, asparagine, ornithine, and the amides, alkyl esters and dipeptidyl derivatives of these amino acids, which medium is substantially free of ammonium ion, provides enhanced quantity and quality of somatic embryos derived from the cultured somatic tissue.

It has also been found that a medium containing ammonium ion and at least one amino acid selected from the group consisting of proline, alanine, arginine, glutamine, lysine, asparagine, serine, ornithine, glutamate and the amides, alkyl esthers and dipeptidyl derivatives of these amino acids in an amount sufficient to stimulate embryogenesis or embryo conversion can provide similar embryo enhancements.

It has been surprisingly discovered that the medium of the present invention provides increased yield of somatic embryos from callus tissue over the typical media used heretofore in the induction, regeneration and maintenance of embryonic tissue. In each stage of these embryogenic procedures, a far greater yield of embryonic tissue can be attained using the media of the present invention than the results previously provided. Furthermore, the advantages obtained through the practice of the present invention have been achieved in a variety of useful plant species including alfalfa, celery, cotton, corn and rice.

Plant cell culture media which can be improved by the practice of the present invention include previously known plant tissue culture media such as those reviewed in Huang, L. and T. Murashige, supra, and in Cloning agricultural plants via in vitro techniques, B.V. Conger,

ed., CRC Press, Inc. pp. 172 et seq. In general, plant culture media provide plant nutrients, sources of energy such as sugar, plant hormones and buffered salts to control the pH and osmotic balance of an aqueous solution.

Representative of such plant cell culture media is the medium known as Schenk and Hildebrandt (SH) medium, Schenk, R.U. and A.C. Hildebrandt, "Medium and techniques for induction and growth of monocotyledonous and dicotyledonous plant cell cultures", Can. J. Bot., 50:199 (1972). As used hereinafter, hormone-free SH medium is the medium disclosed therein including the major salts, vitamins and sucrose, but without the 2,4-D, pCPA and kinetin.

Alternatively, the medium known as Murashige and Skoog (MS), Murashige, T. and F. Skoog, "A revised medium for rapid growth and bioassays with Tobacco tissue cultures", Physiologia Planta., 15:473-497 (1962) can be employed in place of SH medium. As used hereinafter, hormone-free MS medium is the medium disclosed therein including the major salts, vitamins and sucrose, but without the indole-3-acetic acid and kinetin.

The selection of the basic plant cell culture medium to be utilized in the practice of the present invention will be dictated, in part, by the species of plant somatic tissue selected, and is considered to be within the ordinary skill of one experienced in the tissue culture of plant cells and the practice of somatic embryogenesis.

Numerous important crop and horticultural species have been shown to be capable of propagation through tissue culture and somatic embryogenesis. These varieties include, but are not limited to:

Table 1

Vegetable crops	Fruit and nut trees

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alfalfa
asparagus
beet
brussels sprouts
carrot
cauliflower
eggplant
onion
spinach
sweet potato
tomato

Fruit and berries

blackberry
grape
pineapple
strawberry

Foliage

silver vase
begonia
crytanthus
dieffenbachia
dracaena
eiddleleaf
pointsettia
weeping fig
rubber plant

Ferns

Australia tree fern
Boston fern
Maidenhair fern
rabbitsfoot fern
staghorn fern

almond
apple
banana
coffee
date
grapefruit
lemon
olive
orange
peach

Bulbs

lily
daylily
Easter lily
hyacinth

Flowers

African violet
anthurium
chrysanthemum
gerbera daisy
gloxinia
petunia
rose
orchid

Pharmaceutical

atropa
ginseng
pyrethium

Silviculture (forestry)

douglas fir
pine
quaking aspen
redwood
rubber tree

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sword fern

Cereal Grains

barley

corn

millet

pennisetum

wheat

For a more exhaustive list of species capable of somatic embryogenesis, see Evans, D.A. et al., "Growth and Behavior of Cell Cultures: Embryogenesis and Organogenesis" in Plant Tissue Culture: Methods and Applications in Agriculture, Thorpe, ed., Academic Press, page 45 et seq. (1981).

Numerous amino acids are known in the prior art which, with certain exceptions, have the common feature of a free carboxyl group and a free unsubstituted amino group in the α-carbon atom. Proline is a notable exception, since the α-amino group of proline is substituted so that it is in reality an α-imino acid.

Amino acids can be divided generally into protein and nonprotein amino acids wherein protein amino acids include the 20 most commonly recognized. These amino acids include four subgroups: Those with nonpolar or hyrophobic substitutions, including alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan and methionine; amino acids with uncharged polar R groups including serine, threonine, tyrosine, asparagine, glutamine, cysteine and, presumably, glycine; amino acids with negatively charged R groups including aspartic acid and glutamic acid; and amino acids with positively charged R groups including lysine, arginine, and, presumably, histidine.

In addition to the 20 common amino acids, there are numerous others which appear rarely or not at all in

are numerous others which appear rarely or not at all in prot ins. Hydroxylysine and hydroxyproline are rarely found and only in fibrous proteins. Also, over 150 other amino acids are known to occur in different cells and tissues, in free or combined from, which include most commonly citrulline and ornithine, which are intermediates in the synthesis of arginine, as well as numerous β, γ and Δ forms of the common amino acids.

In addition to the basic amino acid structures discussed above, amino acids can be modified in numerous ways without altering their ability to function in the present invention. Among these alterations include the formation of amino acid amides and amino acid alkyl esters by the addition of amino and carboxy groups respectively. In addition, dipeptidyl derivatives of the amino acids can be formed by linking two amino acids through the α-carboxy group and α-amino group. It will be readily appreciated that each pair of amino acids will have two potential dipeptidyl derivatives.

Also of importance to the present invention is the provision of a source of ammonium ion (NH_4^+) to supplement the amino acid-containing media of the present invention. Sources of ammonium ion are also well known in the art of plant tissue culture. Typically, such ammonium ion is provided by way of the inclusion in the medium of a quantity of non-toxic salt of ammonium, formed with an anion which balances the ammonium ion charge, e.g., ammonium chloride, ammonium phosphate or ammonium sulphate. Other sources of ammonium ion are disclosed in Walker, K.A. and S.J. Sato, "Plant Cell Tissue Organ Culture" 1:109-121 (1981).

The following examples are provided in order to illustrate various aspects of the present invention. The examples should not be taken as implying any limitation to

exclusively the claims appended hereto.

Experimental

In general, the methods utilized to practice somatic embryogenesis with plant cell and tissue cultures are well known and require only slight modification for adaptation to a selected plant species. See, for example, Plant Tissue Culture: Methods and Applications in Agriculture, Thorpe, ed., supra (1981).

For example, alfalfa, embryogenesis can be routinely induced in the Regen S line of Saunders and Bingham, "Production of Alfalfa Plants from Callus Tissue," Crop Sci., 12:804-808 (1972).

Plants of Medicago sativa cultivar Regen S derived from the second cycle recurrent selection for regeneration from the cross of the varieties Vernal and Saranac were used. Callus was initiated by surface sterilizing petioles with 50% Clorox^R for five minutes, washing with H_2O and plating on hormone-free SH medium, containing the salts, vitamins and sucrose of Schenk-Hildebrandt medium (Schenk, R.U. and A.C. Hildebrandt, supra, (1972)). The medium contained 25 μM α-naphthyleneacetic acid and 10 μM kinetin and 0.8% (w/v) agar (termed maintenance medium). Callus which formed on the explant tissue was separated from the remaining uncallused tissue and repeatedly subcultured on maintenance medium. Callus was subcultured at 3 week intervals and grown under indirect light at 27°C.

Three to nine grams of callus was collected at 17 to 24 days post-subculture from plates of maintenance medium and transferred to 100 ml of liquid SH containing 50 μM 2,4-dichlorophenoxyacetic acid (2,4-D) and 5 μM kinetin (B) for induction. Walker, K.A., M.L. Wendeln and E.G. Jaworski, Plant Sci. Lett. 16:23-30 (1979). Cells were cultured in 500 ml flasks for 3 days at 27°C on an

orbital shaker at 100 R.P.M. under indirect light.

Induced cells were asceptically sized on a series of column sieves (Fisher Scientific) under gentle vacuum. Cell clumps either fell or were forced through a 35 mesh (480 μm) and collected on a 60 mesh (230 μm) through stainless steel screen. Cells retained on the 60 mesh screen were washed with 500 ml of SH minus hormone medium for every 100 ml of induction culture volume. The washing medium was removed by vacuum. The fresh weight of the cell clumps was taken and cells were resuspended in SH medium without hormones at 150mg fresh weight per ml. Seventy-five mg (0.5ml) of resuspended cells were pipeted onto approximately 10 ml of agar solidified medium in 60mm x 15 mm petri dishes.

Alternatively, somatic embryogenesis in suspension culture will occur if 300 mg (2ml) of resuspended cells are delivered to 8 ml of hormone-free liquid SH medium contained in a 50 ml erlenmeyer flask. The embryogenesis media contained SH medium (NH_4^+ equal to 2.6mM with 3% (w/v) sucrose without hormones. Ammonium ion free medium was made by substituting an equivalent amount of NaH_2PO_4 for the $NH_4H_2PO_4^+$ of SH. The 25mM NH_4^+ control medium consisted of ammonium free medium supplemented with 12.5mM $(NH_4)_2SO_4$. All organic and inorganic sources of reduced nitrogen were sterilized by 0.2 μm filtration and subsequently added to freshly autoclaved medium.

Each treatment was generally plated in 10 replicates. Dishes were parafilm wrapped and incubated for 21 days. Suspension flasks were foam plugged, sealed with Saran Wrap® and incubated for 14 days on an orbital shaker at 100 rpm. Incubation was at 27°C under 12 hour illumination from cool white fluorescent tubes at 28 cm from solidified cultures or 200 cm from suspension

cultures.

Embryogenesis was visually measured after incubation by counting green centers of organization on the callus using a stereo microscope at a magnification of 10X. Embryo size was measured using a calibrated ocular scale at 10X magnification. Embryo shape was determined by visual examination. Conversion of embryos to whole plants with root and shoot axis (first primary leaf) was done by aseptically transferring embryos from amino acid treatments at 21 days of initial culture to half-strength hormone-free SH medium supplemented with 25 μM gibberelic acid and 0.25 μM α-naphthyleneacetic acid solidified with 0.8% agar.

1. Somatic Embryogenesis in Media Containing Ammonium

A. Leguminosae somatic cell culture

As a representative of the Leguminosae family, alfalfa tissue, Medicago sativa, Regen S was cultured as outlined above and assayed for somatic embryogenesis in culture medium containing 2.6 mM ammonium in accordance with the present invention. All protein amino acids were tested at between 1 and 100 mM concentrations. Two response types emerged from this initial screen and based on these results further tests with sieved cells were performed. Table 2 excludes amino acids of the first response type, those which were found to be toxic to growth or inhibitory to embryogenesis compared to the SH-medium (2.6mM NH_4^+) control. These amino acids included the sulfur and aromatic and most of the branched chain family. None of these amino acids stimulated embryogenesis over the SH medium control and all were toxic, in that they inhibited growth or caused browning of the callus either at 1 or 10 mM.

The second response type from the initial screen either stimulated embryogenesis or caused an increase in

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embryo size when compared to the SH control. See Table 2. Detailed concentration dependence studies were performed on these amino acids and the results are shown in Figure 1. The amino acid most effective in stimulating somatic embryo formation was proline, which yielded nearly 3-fold more embryos than the 2.6 mM NH_4^+ control and was twice as effective as 25 mM NH_4^+, the optimal ammonium concentration in alfalfa (D). (Walker, et al., supra). Alanine, arginine, glutamine and lysine were all less effective but stimulated embryo formation to approximately the level of 25 mM NH_4^+. Serine and asparagine showed less stimulation of embryogenesis compared to the SH control, but increased embryo size.

Table 2 summarizes the amino acids and other nitrogen sources which have been found to be stimulatory to somatic embryogenesis in alfalfa. It is important to note that the ester and amide forms of proline are highly active in stimulating embryo numbers and quality as is the dipeptide, prolyl alanine. It is interesting to note that the nonprotein amino acid ornithine is also active.

Table 2

The Effect of Reduced Nitrogen Sources on Somatic Embryogenesis in Alfalfa

Stimulatory Sources
Ammonium
Proline
Alanine
Glutamine
Arginine
Asparagine
Ornithine
Serine
Lysine

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L-proline amide

L-prolyl-L-alanine

L-proline methyl ester

Using the techniques described above, embryo quality was measured by visual inspection of the embryo size. The data is presented in Table 3.

Table 3

Effect of Reduced Nitrogen Treatment on Somatic Embryo Size

Treatment	Length	Width
Control (25mM NH_4^+)	0.805 ± 0.084	0.383 ± 0.019
100mM L-Proline	1.143 ± 0.081	0.867 ± 0.046
30mM L-Alanine	1.163 ± 0.090	0.744 ± 0.037
100mM L-Alanine	1.192 ± 0.102	0.833 ± 0.049
30mM L-Arginine	1.521 ± 0.142	0.663 ± 0.048
30mM L-Glutamine	1.342 ± 0.122	0.773 ± 0.051
3mM L-Lysine	1.163 ± 0.096	0.652 ± 0.039
3mM L-Asparagine	0.699 ± 0.062	0.446 ± 0.027
10mM L-Asparagine	1.239 ± 0.102	0.610 ± 0.034

Based on the data of Table 2 and Table 3, the effectiveness of the amino acid additives in improving embryo size can be ranked in the following order:

Arginine ≥ glutamine > alanine > proline > NH_4^+.

Using the techniques described above, the conversion of embryos to whole plants with root and shoot axis (first primary leaf) was observed and tabulated. The results are as follows:

Table 4

Conversion of Somatic Embryos to Alfalfa Plantlets

Initial Treatment	% Plants with First primary Leaf
25 mM NH_4^+	33.3% + 4.2
100 mM L-Proline	54.0% + 6.4

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50 mM L-Alanine	63.5% + 4.4
30 mM L-Arginine	59.0% + 6.2
30 mM L-Glutamine	67.0% + 3.4

Based on the data of Table 4, the effectiveness of an additive on the conversion of embryos to plantlets is as follows:

Glutamine > alanine ≥ arginine > proline > NH_4^+

From the correlation between these sets of data it is shown that embryo size is a good indicator of embryo conversion to plantlets and thus a good indicator of the quality of embryos produced by a given technique.

The optimal amounts of added amino acids were determined for the stimulation of somatic embryogenesis on both agar solidified cultures and liquid suspension cultures. These data are presented in Tables 5 and 6 as follows:

Table 5

Stimulation of Alfalfa Somatic Embryogenesis by Amino Acid Additions to Agar Solidified Culture in the Presence of 2.6 mM NH_4^+

Source	% Maximal Stimulation	Concentration Range in mM	Concentration Optimum in mM
Control (2.6 mM NH_4^+)	100	---	---
L-Proline	330	10 to 300	(100)
L-Alanine	314	20 to 150	(75-100)
L-Glutamine	175	20 to 50	(30-40)
L-Arginine	244	5 to 50	(30-40)
L-Asparagine	155	0.5 to 3	(1)
L-Ornithine	156	1 to 3	(1-3)
L-Serine	160	0.5 to 2	(1)
L-Lysine	233	1 to 10	(3)
L-Proline amide	240	30 to 200	(50-100)
L-Proline methyl ester	241	5 to 25	(10)

L-Prolyl-L-alanine	210	30 to 200	(50-100)

Table 6

Stimulation of Alfalfa Somatic Embryogenesis by Amino Acid Additions in Liquid Suspension Cultures in the Presence of 2.6 mM NH_4^+

Source	% Maximal Stimulation	Concentration Range in mM	Concentration Optimum in mM
Control (2.6 mM NH_4^+)	100		
L-Proline	528	10 to 300	(100)
L-Alanine	240	25 to 200	(50)
L-Glutamine	243	5 to 75	(50)
L-Arginine	168	5 to 75	(50)
L-Lysine	180	1 to 10	(3)

B. Umbelliferae somatic cell culture

As a representative of the Umbelliferae family, seeds of celery, Apium graveolens (variety Calmario) were germinated for one to two weeks. The resulting seedlings were sterilized with a solution of 10% Clorox^R for 20 minutes. Cotyledons or hypocotyls were removed and explants were placed on 0.8% agar solidified hormone-free SH medium containing 25 μM 2,4-D and 5 μM benzyladenine. After initiation of callus (3-4 weeks), callus was transferred to SH medium with 2.5 μM 2,4-D and 0.5 μM kinetin. Heat labile additives were filter sterilized and added to warm medium. When required, specific amounts of tissue for innoculation were obtained using a modified spatula device and filling this to uniform volume. Subsequent subcultures of callus were on SH medium plus 1 μM picloram and 0.5 μM benzyladenine. For somatic embryo production 75 mg of callus cells was transferred to 0.8% agar solidified hormone-free SH medium containing filter sterilized additives and incubated for 18 to 30 days at

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24°C under the same conditions as alfalfa.

Amino acids proline, alanine and glutamine were compared against NH_4^+ control-treated embryos. Treating cultures with 50 mM alanine resulted in higher frequency embryogenesis than all other treatments, as well as embryos which had better cotyledons, root and primary leaf development than other cultures. The following order of total embryo numbers formed was observed:

20-100 mM alanine > 50 mM proline >

25 mM glutamine-N > 25 mM NH_4^+

Although proline stimulated embryogenesis better than glutamine, the latter resulted in better development of seedling-like embryos. Ammonium treated cultures developed smaller and fewer embryos than all other treatments. Glutamic acid, when added singly to celery regeneration medium at 30 mM, stimulates embryo number in celery compared to 25 mM NH_4^+ - treated material. Alanine, proline, glutamine and glutamate at the above concentrations improve celery embryo conversion to plantlets compared to NH_4^+ treated embryos.

C. Gramineae Somatic Cell Culture

As a species representative of the Gramineae family, Zea mays somatic embryogenesis was performed employing media in accordance with the present invention.

Ears of corn at ten days post fertilization were harvested and immature embryos were dissected from these aseptically. Embryos were placed onto N-6 mineral salt medium (Chu, C.C., Wang, C.C., Sun, C.S., Hsu, C., Yin, K.C. and Chu, C.Y., 1975. Establishment of an efficient medium for anther culture of rice through comparative experiments on the nitrogen sources. Sci. Sin. 16, 659-688) plus 3% sucrose and 5 μM 2,4-D for 21 days. After incubation callus was scored for formation of embryo

masses on each callus formed while in the presence or absence of L-proline. The results were as indicated in Table 7, where, the percent response is the average frequency of embryo formation of between 287 and 1165 replicate embryo explants.

Table 7. Effect of L-Proline on Embryo Callus Formation in Corn.

Proline Conc. (mM)	% Embryo Callus Formation
0	15.8
6	20.6
12	20.8
24	20.2

As a further example of somatic embryogenesis involving the Gramineae, the rice species Oryza sativa was regenerated in accordance with the practice of the present invention.

Seeds of Oryza sativa were dehusked, surface sterilized and placed on Murashige and Skoog (MS) salts (Murashige, T. and F. Skoog. (1962), supra) plus 4% sucrose, 0.26 mM tryptophan, 5 μM 2,4-D, 1 μM kinetin, pH 6.2 with 2.5g/1 Gelrite as a gelling agent. After 15 to 21 days individual seeds were scored for embryo formation on the scutellar region of the seed with a dissecting microscope. Treatments treated with and without L-proline are noted in Table 8.

Table 8. Effect of L-Proline on Embryo Formation in Rice Callus Cultures

Proline Conc. (mM)	% Embryo Formation
0	57.8
3	59.0

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10	77.8
30	64.9
50	70.1
100	68.4

D. Malvaceae Somatic Cell Culture

As an example of the practice of the present invention in plant somatic tissue of the Malvaceae family, cultures of two cotton species were regenerated in media prepared in accordance with the present disclosure.

Gossypium hirsutum. Cultures initiated from surface sterilized seed of Gossypium hirsutum were subcultured on Murashige and Skoog salts plus 3% sucrose and 0.5 μM NAA, 5 μM 2-isopentanyladenine with 0.8% agar medium for four week subcultures. Cultures were induced for 10 days to form embryos on medium containing either 0.5 μM 2,4-D plus 0.2 μM kinetin or 1 μM NAA and 0.5 μM kinetin with or without proline in liquid suspension culture. Cells were then transferred to hormone-free SH medium with 3% sucrose and 10 mM L-glutamine for regeneration. After four weeks cultures were evaluated for formation of embryos with mature cotyledons. The results were as indicated in Table 9.

Table 9. Effect of Added Proline on the Regeneration of Cotyledonary Cotton (Gossypium hirsutum) Embryos in Suspension Culture

Proline Conc (mM)	Embryos with Cotyledons
0	5.5
24	12.5

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Gossypium klotzschianum. Cultures initiated from surface sterilized seed were subcultured on Murashige and Skoog salts, 3% sucrose, 0.5 μM NAA and 5 μM 2-isopentanyladenine. Callus was suspended for 10 days in MS salts, 3% sucrose and 0.2 μM picloram prior to regeneration for 21 days on hormone-free medium with added L-glutamine. The results were as indicated in Table 10.

Table 10. Effect of L-Glutamine on Embryo Formation in Gossypium klotzschianum

L-Glutamine Conc. (mM)	Cotyladonary Embryos
5	0
10	2.0
20	3.5

2. Amino Acid Interaction With Sources of Ammonium Ion

Cells were induced, sieved and plated as in the above experiments. The concentrations of proline or arginine and NH_4^+ were varied to determine if the optimum concentration for any additive alone was influenced by the presence of the additional additive.

1. Proline: Proline was tested over a range of 30 mM to 300 mM where the amount of added NH_4^+ varied between 0 and 25 mM. The results are indicated in Table 11.

2. Arginine: A similar experiment where the concentration of arginine was varied in addition to the concentration of NH_4^+ added to the medium. The results are shown in Table 11.

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Table 11

Effect of Amino Acid Interaction with Ammonium Ion in Alfalfa (mean number of embryos produced in at least seven trials)

Proline Concentration (mM)	30	100	300
NH_4^+ Concentration (mM)			
0	326	470	133
1.0	502	747	541
2.6	753	731	825
10.0	887	811	572
25.0	744	1,0472	844

Arginine Concentration (mM)	0	10	30	100
NH_4^+ Concentration (mM)				
0	12	147	126	99
1.0	70	252	246	157
2.6	207	298	306	264
10.0	340	408	411	311
25.0	335	297	233	148

It is seen in each case that a synergistic effect resulted when the optimum amounts of arginine or proline and optimum amounts of NH_4^+ were added.

In a repeat of a portion of the Example portrayed in Table 11, various concentrations of NH_4^+ and L-proline were tested for their effect on somatic embryo quantity and conversion to plantlets.

Table 12

Effect of Ammonium and Proline on Somatic Embryo Quantity and Quality

Proline (mM)	NH_4^+ (mM)	SE Quantity	% Conversion
0	0	4 ± 1	3 ± 3
0	2.6	36 ± 5	4 ± 3

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0	25	60 ± 6	9 ± 5
10	2.6	95 ± 12	25 ± 5
30	2.6	134 ± 17	42 ± 7
100	2.6	175 ± 19	42 ± 4
30	10	187 ± 24	42 ± 6

It is seen that proline plus ammonium media improve embryo quantity and that proline improves embryo quality in the presence of high or low ammonium.

3. Amino Acid Additions to Media Substantially Free of Ammonium Ion

Alfalfa cells were induced and plated as in the above examples, except that NH_4^+ was deleted from the media formulation. A range of amino acid concentrations was tested for their effect on somatic embryogenesis and the results are summarized in Table 13.

Table 13

Stimulation of Alfalfa Somatic Embryogenesis by Amino Acid Additions to Agar Solidified Cultures in Media Substantially Free of NH_4^+

Addition	% Maximal Stimulation	Concentration Range in mM	Concentration Optimum in mM
Control without NH_4^+)	100	---	---
L-Proline	525	6 to 300	(10-30)
L-Arginine	1,200	1 to 100	(20-50)
L-Asparagine	750	1 to 100	(2-10)
L-Ornithine	900	0.3 to 3	(1)
L-Lysine	500	1 to 10	(3)

In the substantial absence of NH_4^+, the above amino acids are shown to stimulate somatic embryogenesis as the sole reduced nitrogen source in culture media. In addition, with the exception of ornithine, substantial

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improvements in the quality of the produced embryos, as determined by size, shape and degree of maturation, were obtained by the above additions throughout the stated concentration ranges.

4. Combinations of Amino Acids

The following Table shows the effect of adding combinations of amino acids to alfalfa cultures in the absence of NH_4^+. Combinations of amino acids have a synergistic effect on embryo numbers.

Table 14

		Embryo Number
Expt. 1	50 mM L-Proline	80
	30 mM L-Glutamine	50
	50 mM Proline and 30 mM Glutamine	248
Expt. 2	100 mM L-Proline	27
	100 mM L-Alanine	74
	100 mM L-Proline+50mM L-Alanine	215
	30 mM L-Arginine	135
	100 mM Proline + 30 mM Arginine	215

The largest and highest quality embryos were observed in treatments containing proline combined with other amino acids.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious to one skilled in the art that certain changes and modifications may be practiced within the scope of the appended claims.

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THE EMBODIMENTS OF THE INVENTION IN WHICH AN EXCLUSIVE PROPERTY OR PRIVILEGE IS CLAIMED ARE DEFINED AS FOLLOWS:

1. A method for producing embryonic tissue from Gramineae, Umbelliferae or Malvaceae somatic tissue, wherein somatic tissue is regenerated from induced cells in a nutritive plant cell culture medium to form embryonic tissue, said method comprising:

providing a nutritive plant cell culture medium comprising:

a medium having all mineral salts, vitamins and nutrients required to maintain tissue viability, together with a source of ammonium ion; and

an addition to said medium of at least one amino acid component selected from the group consisting of L-proline, L-arginine, L-lysine, L-asparagine, L-serine, L-ornithine and the amide, alkyl ester and dipeptidyl derivatives thereof,

upon which somatic embryos can differentiate from induced cells, the ammonium and amino acid components of said medium provided in amounts sufficient to increase the quantity or improve the quality of somatic tissue to be produced;

exposing undifferentiated plant somatic tissue selected from the group consisting of Gramineae, Umbelliferae and Malvaceae somatic tissue to said culture medium to cause embryogenesis in said plant somatic tissue to form somatic embryos; and

sustaining said somatic embryos on said culture medium to permit embryo development into plantlets.

2. A method for producing embryonic tissue according to claim 1 wherein said medium addition is selected from the group consisting of L-proline, its amide, alkyl ester and dipeptidyl derivatives, added to said medium in an amount sufficient to provide a final concentration of L-proline in the medium greater than 1mM to approximately 300mM.

3. A method for producing embryonic tissue according to claim 1 wherein said medium addition is selected from the group consisting of L-arginine, its amide, alkyl ester and dipeptidyl derivatives, added to said medium in an amount sufficient to provide a final concentration of L-arginine in the medium of approximately 3 to 75mM.

4. A method for producing embryonic tissue according to claim 1 wherein said medium addition is selected from the group consisting of L-lysine, its amide, alkyl ester and dipeptidyl derivatives, added to said medium in an amount sufficient to provide a final concentration of L-lysine in the medium of approximately 1 to 10mM.

5. A method for producing embryonic tissue according to claim 1 wherein said medium addition is selected from the group consisting of L-asparagine, its amide, alkyl ester and dipeptidyl derivatives, added to said medium in an amount sufficient to provide a final concentration of L-asparagine in the medium of approximately 0.5 to 3mM.

6. A method for producing embryonic tissue according to claim 1 wherein said medium addition is selected from the group consisting of L-serine, its amide, alkyl ester and dipeptidyl derivatives, added to said medium in an amount sufficient to provide a final concentration of L-serine in the medium of approximately 0.5 to 2mM.

7. A method for producing embryonic tissue according to claim 1 wherein said medium addition is selected from the group consisting of L-ornithine, its amide, alkyl ester and dipeptidyl derivatives, added to said medium in an amount sufficient to provide a final concentration of L-ornithine in the medium of approximately 1 to 3mM.

8. A method for producing embryonic tissue according to claim 1 wherein said medium addition is selected from the group of amino acid components consisting of L-proline amide and its dipeptidyl derivatives containing L-proline amide, added to said medium in an amount sufficient to provide a final concentration of L-proline amide in the medium of approximately 1 to 200mM.

9. A method for producing embryonic tissue according to claim 1 wherein said medium addition is selected from the group of amino acid components consisting of L-proline methyl ester and its dipeptidyl derivatives containing L-proline methyl ester, added to said medium in an amount sufficient to provide a final concentration of L-proline methyl ester in the medium of approximately 0.3 to 40mM.

10. A method for producing embryonic tissue according to claim 1 wherein the source of ammonium ion is selected from the group of ammonium containing compounds consisting of ammonium chloride, ammonium nitrate, ammonium carbonate, ammonium sulfate, ammonium phosphate and ammonium citrate.

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11. A method for producing embryonic tissue according to claim 10 wherein the source of ammonium ion is provided in an amount sufficient to provide a final concentration of ammonium ion in the medium of approximately 0.5 to 50mM.

12. A method for producing embryonic tissue according to claim 1 wherein said medium is selected from the group consisting of Schenk-Hildebrandt medium and Murashige and Skoog medium.

13. A method of producing embryonic tissue from Gramineae, Umbelliferae or Malvaceae somatic tissue, wherein somatic tissue is regenerated from induced cells in a nutritive plant cell culture medium to form embryonic tissue, said method comprising:

providing a nutritive plant cell culture medium comprising all mineral salts, vitamins and nutrients required to maintain tissue viability, which medium is substantially free of ammonium ion, together with an addition to said medium of L-glutamine and at least one amino acid component selected from the group consisting of L-proline, L-arginine, L-lysine, L-asparagine, L-serine, L-ornithine, the amide, alkyl ester and dipeptidyl derivatives thereof, upon which somatic embryos differentiate from induced cells, **the amino acid components of said medium provided in amounts sufficient to increase the quantity or improve the quality of somatic tissue to be produced;**

exposing undifferentiated plant somatic tissue selected from the group consisting of Gramineae, Umbelliferae and Malvaceae somatic tissue to said culture medium to cause embryogenesis in said plant somatic tissue to form somatic embryos; and

sustaining said somatic embryos on said provided nutritive plant cell culture medium to thereby permit embryo development into plantlets.

14. A method for producing embryonic tissue according to claim 13 wherein said L-glutamine is provided in an amount to provide a final concentration of L-glutamine in said medium of approximately 10 to 50mM.

15. A method for producing embryonic tissue according to claim 13 wherein said medium addition is selected from the group consisting of L-proline, its amide, alkyl ester and dipeptidyl derivatives, added to said medium in an amount sufficient to provide a final concentration of L-proline in the medium greater than 1mM to approximately 300mM.

16. A method for producing embryonic tissue according to claim 13 wherein said medium addition is selected from the group consisting of L-arginine, its amide, alkyl ester and dipeptidyl derivatives, added to said medium in an amount sufficient to provide a final concentration of L-arginine in the medium of approximately 3 to 75mM.

17. A method for producing embryonic tissue according to claim 13 wherein said medium addition is selected from the group consisting of L-lysine, its amide, alkyl ester and dipeptidyl derivatives, added to said medium in an amount sufficient to provide a final concentration of L-lysine in the medium of approximately 1 to 10mM.

18. A method for producing embryonic tissue according to claim 13 wherein said medium addition is selected from the group consisting of L-asparagine, its amide, alkyl ester and dipeptidyl derivatives, added to said medium in an amount sufficient to provide a final concentration of L-asparagine in the medium of approximately 0.5 to 3mM.

19. A method for producing embryonic tissue according to claim 13 wherein said medium addition is selected from the group consisting of L-serine, its amide, alkyl ester and dipeptidyl derivatives, added to said medium in an amount sufficient to provide a final concentration of L-serine in the medium of approximately 0.5 to 2mM.

20. A method for producing embryonic tissue according to claim 13 wherein said medium addition is selected from the group consisting of L-ornithine, its amide, alkyl ester and dipeptidyl derivatives, added to said medium in an amount sufficient to provide a final concentration of L-ornithine in the medium of approximately 1 to 3mM.

21. A method for producing embryonic tissue according to claim 13 wherein said medium addition is selected from the group of amino acid components consisting of L-proline amide and its dipeptidyl derivatives containing L-proline amide, added to said medium in an amount sufficient to provide a final concentration of L-proline amide in the medium of approximately 1 to 200mM.

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22. A method for producing embryonic tissue according to claim 13 wherein said m dium addition is selected from the group of amino acid components consisting of L-proline methyl ester and its dipeptidyl derivatives containing L-proline methyl ester, added to said medium in an amount sufficient to provide a final concentration of L-proline methyl ester in the medium of approximately 0.3 to 40mM.

23. A method for producing embryonic tissue according to claim 13 wherein said medium is selected from the group consisting of Schenk-Hildebrandt medium and Murashige and Skoog medium.

24. A method for producing embryonic tissue from Gramineae, Umbelliferae or Malvaceae somatic tissue, wherein somatic tissue is regenerated from induced cells in a nutritive plant cell culture medium to form embryonic tissue, said method comprising:

providing a nutritive plant cell culture medium comprising all mineral salts, vitamins and nutrients required to maintain tissue viability, together with an addition to said medium of at least one amino acid component selected from the group consisting of

a) L-proline, its amide, alkyl ester and dipeptidyl derivatives, added in an amount sufficient to provide a final concentration of L-proline in the medium of greater than 10mM to approximately 300mM;

b) L-arginine, its amide, alkyl ester and dipeptidyl derivatives, added in an amount sufficient to provide a final concentration of L-arginine in the medium of approximately 15mM to approximately 100mM;

c) L-lysine, its amide, alkyl ester and dipeptidyl derivativ s, added in an amount sufficient to provide a final concentration of L-lysine in the medium of approximately 1mM to approximately 10mM;

d) L-asparagine, its amide, alkyl ester and dipeptidyl derivatives, added in an amount sufficient to provide a final concentration of L-asparagine in the medium of approximately 30mM to approximately 100mM;

e) L-ornithine, its amide, alkyl ester and dipeptidyl derivatives, added in an amount sufficient to provide a final concentration of L-ornithine in the medium of approximately 0.3mM to approximately 3mM;

f) L-proline amide and its dipeptidyl derivatives containing L-proline amide, added in an amount sufficient to provide a final concentration of L-proline amide in the medium of approximately 1mM to approximately 200mM; and

g) L-proline methyl ester and its dipeptidyl derivatives containing L-proline methyl ester, added in an amount sufficient to provide a final concentration of L-proline methyl ester in the medium of approximately 0.3mM to approximately 40mM,

upon which medium somatic embryos can differentiate from induced cells;

exposing undifferentiated plant somatic tissue selected from the group consisting of Gramineae, Umbelliferae and Malvaceae somatic tissue to said culture medium to cause embryogenesis in said plant somatic tissue to form somatic embryos; and,

sustaining said somatic embryos on said culture medium to thereby permit embryo development into plantlets.

25. A method for producing embryonic tissue according to claim 24 wherein said medium is selected from the group consisting of Schenk-Hildebrandt medium and Murashige and Skoog medium.

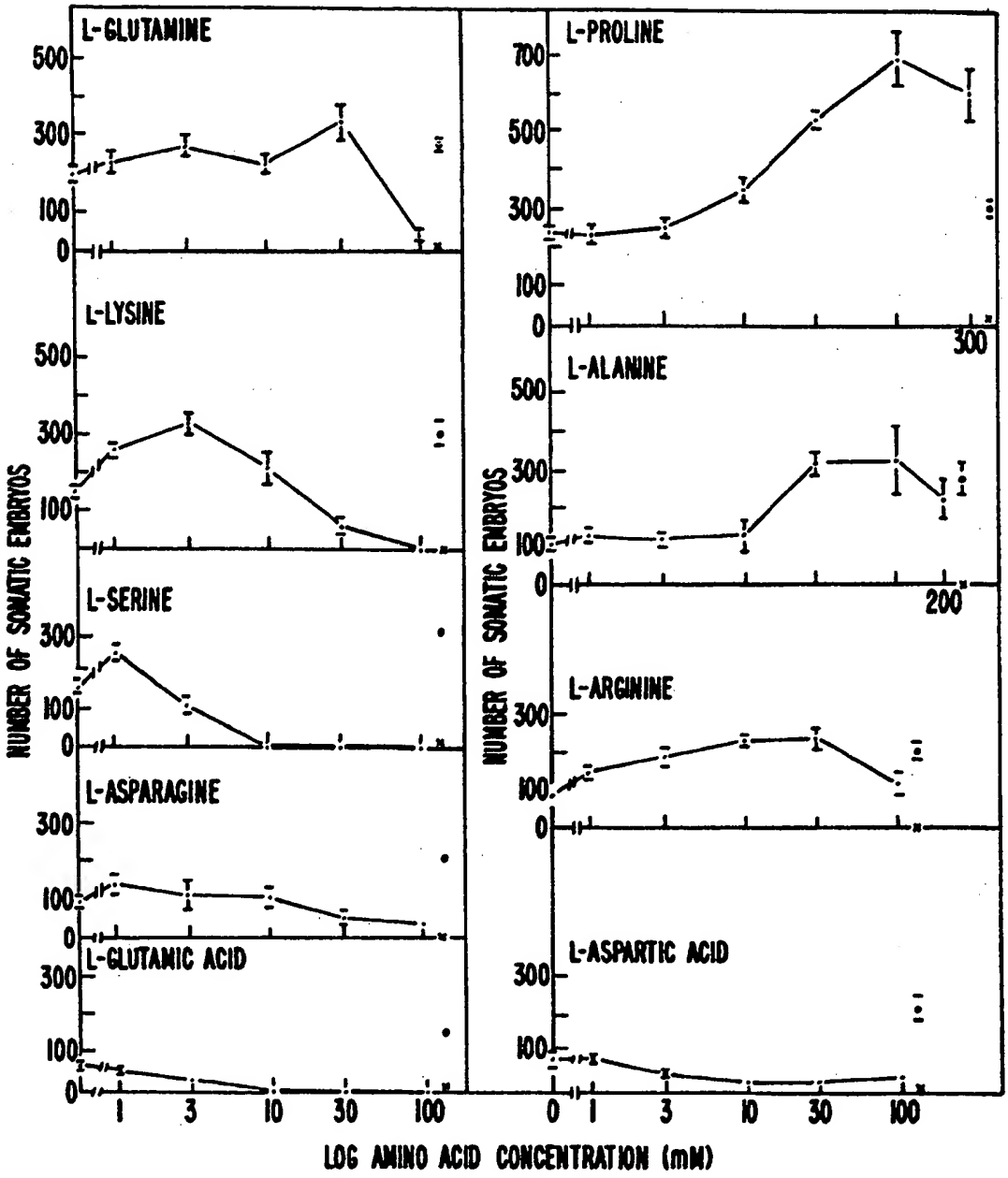
L-GLUTAMINE
L-PROLINE
L-LYSINE
L-ALANINE
L-SERINE
L-ARGININE
L-ASPARAGINE
L-GLUTAMIC ACID
L-ASPARTIC ACID
NUMBER OF SOMATIC EMBRYOS
LOG AMINO ACID CONCENTRATION (mM)
0
1
3
10
30
100
200
300
500
700

FIG.__1.

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